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Advances in Brief

Taxol Induces *bcl-2* Phosphorylation and Death of Prostate Cancer Cells[1]

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Abstract

Treatment of prostate cancer cell lines expressing *bcl-2* with taxol induces bcl-2 phosphorylation and programmed cell death, whereas treatment of *bcl-2*-negative prostate cancer cells with taxol does not induce apoptosis. bcl-2 phosphorylation seems to inhibit its binding to *bax* since less *bax* was observed in immunocomplex with bcl-2 in taxol-treated cancer cells. These findings support the use of the anticancer drug taxol for the treatment of *bcl-2*-positive prostate cancers and other *bcl-2*-positive malignancies, such as follicular lymphoma.

Introduction

The *bcl-2*[3] gene has been isolated by taking advantage of its juxtaposition to the immunoglobulin heavy chain locus in follicular lymphoma (1, 2). It codes for a protein of M_r 26,000 with a hydrophobic carboxyl terminus that is associated with all cellular membranes (3–7). It has been shown that the bcl-2 protein promotes cell survival (8) by inhibiting the process of programmed cell death or apoptosis (9, 10). It is not clear, however, what biochemical mechanisms are involved in such inhibition. *bcl-2* is expressed not only in lymphoid cells but is also expressed in a large variety of tissues and cell types. Recently, it has been shown that hormone therapy-resistant prostate cancers often express *bcl-2* (11), whereas the prostate cells from which prostate cancers originate are *bcl-2* negative (12). Similarly, other carcinomas resistant to a variety of anticancer drugs express *bcl-2*, suggesting that *bcl-2* may protect cancer cells from programmed cell death induced by a variety of anticancer agents (13, 14). We have shown previously that human leukemic cells exposed to phosphatase inhibitors express a phosphorylated form of bcl-2 and die, suggesting that phosphorylation of bcl-2 may inhibit *bcl-2* function (15). In that study, we have also shown that treatment of the leukemic cells with taxol leads to bcl-2 phosphorylation (15).

Because prostate cancers that are refractory to conventional antiandrogen therapy often express *bcl-2*, we investigated the effect of the anticancer drug taxol on the viability of prostate cancer cells and the phosphorylation of bcl-2.

Materials and Methods

Tumor Cells. The human hormonal-independent DU145 and PC-3 cell lines and hormonal-dependent LNCaP prostatic carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). All cell lines were grown in humidified atmosphere at 37°C in 5% CO_2. These tumor cell lines were maintained in culture as adherent cells in RPMI 1640 containing 10% FCS and gentamycin.

Reagents. Taxol, etoposide, and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal bcl-2 antibody was obtained from Genosys (Woodland, TX). Enhanced chemiluminescence Western blot detection reagents were purchased from Amersham (Arlington Heights, IL). Stock solutions of drugs were usually prepared in DMSO and diluted with PBS or medium.

Immunoblot Analysis. Cells were lysed as described previously (15). Equivalent amounts of protein from each sample were electrophoresed by 5–15% gradient SDS-PAGE. Proteins were transferred on nitrocellulose. Western blotting was carried out by the methods described earlier (5, 9, 15).

DNA Fragmentation Assay. A pellet of 10^7 cells was resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100, pH 8.0) containing 100 μg/ml proteinase K and incubated at 37°C for 4–6 h. DNA extraction was carried out with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v). The purified samples were evaporated to a volume of 0.2 ml using a Speed-Vac (Savant, Farmingdale, NY). The final concentration of DNA was determined by UV absorbance at 260 nm. DNA (10 μg/lane) was electrophoresed on 1.8% agarose gels containing ethidium bromide (1 μg/ml).

Development of *bax* Peptide Antibody. Peptide antibody against *bax* was developed by immunizing the rabbit with a synthetic peptide from the 46–66 amino acid region of human bax protein. Peptide conjugation was carried out using Imject maleimide-activated keyhole limpet hemocyanin (Pierce Chemical Co., Rockford, IL).

Results and Discussion

As shown in Fig. 1*A*, treatment of the PC3 prostate carcinoma cell line with taxol at the concentration of 5–10 μM for 24 h results in the expression of two slower-migrating (phosphorylated) forms of bcl-2 protein and in apoptotic cell death as determined by DNA fragmentation (laddering; Fig. 2). The slower-migrating forms of *bcl-2* have been shown by studies published previously (15) to represent phosphorylated forms of the protein. As shown in Fig. 1*B*, the induction of the phosphorylated form of *bcl-2* occurs after 4 h of treatment at the concentration of 10 μM. Treatment of a different prostate cancer cell line, LNCaP, that also expresses *bcl-2* results in phosphorylation of bcl-2 (Fig. 1*C*) and in cell death (Fig. 2). Interestingly, the DU145 prostate cancer cell line that does not express *bcl-2* (Fig. 1*D*) is insensitive to the apoptotic effect of taxol (Fig. 2). The insensitivity of the apoptotic effect of taxol in DU145 cells could be due to the resistance specific for taxol. But to our knowledge, no information is available in the literature in regard to taxol resistance of DU145 cells. In all, these results suggest that the expression of *bcl-2* may render the prostate cancer cells sensitive to taxol-induced apoptosis through its phosphorylation. The additional experimentation is needed to establish a direct functional connection between bcl-2 phosphorylation and apoptosis. Thus phosphorylated bcl-2 could represent a positive signal for induction of apoptotic death. It is worth mentioning that the exposure of cancer cells to etoposide, a topoisomerase II inhibitor, did not induce phosphorylation of bcl-2 protein, and the cells did not undergo apoptosis (data not shown). At present, we do not know whether prostate cancer cells acquired resistance to this drug through classical mechanisms. Further studies using other dissimilar anticancer drugs are in progress in the laboratory.

We have also carried out subcellular fractionation experiments to attempt to determine whether there is a difference in the expression of phosphorylated bcl-2 in different subcellular fractions. As shown in Fig. 3, we observed the phosphorylated form of bcl-2 induced by exposure to taxol predominantly in the microsomal fraction.

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[3] The abbreviation used is: *bcl-2*, B-cell leukemia/lymphoma-2 gene.

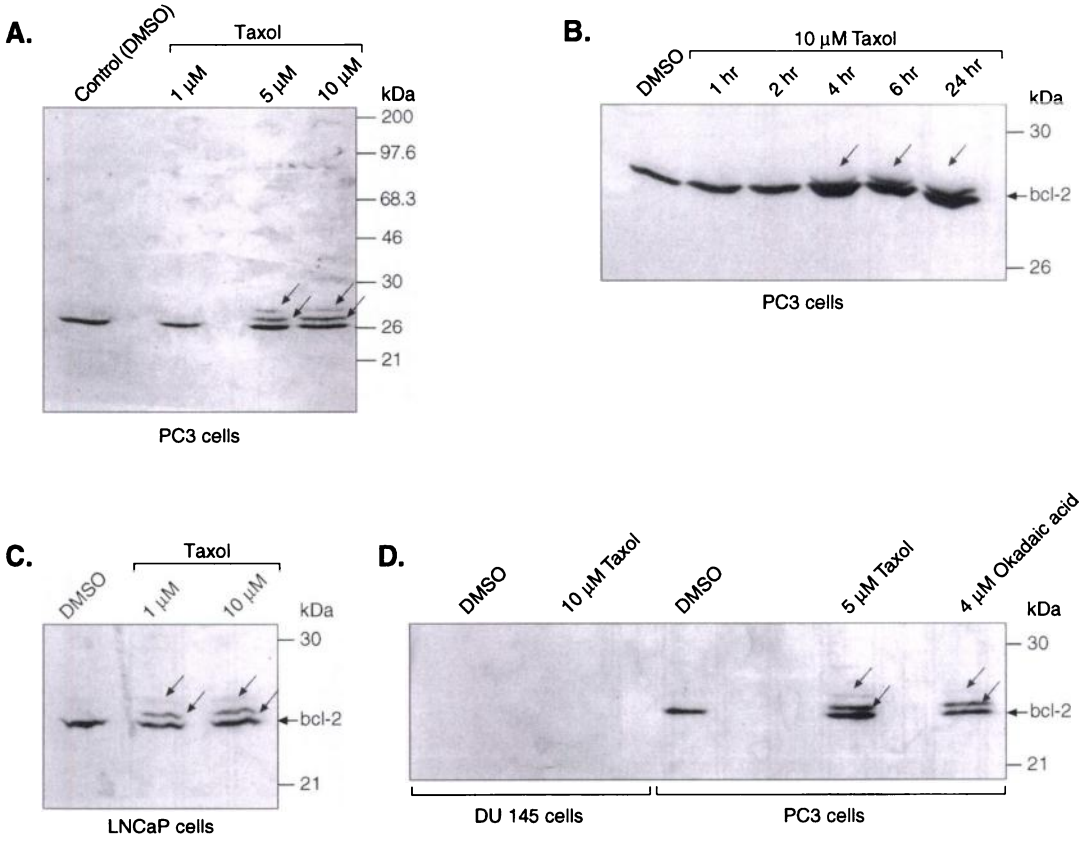

Fig. 1. Taxol induces phosphorylation of bcl-2 protein in prostate cancer cells. *A*, immunoblot analysis of total protein extract from DMSO or taxol-treated prostate cancer cells PC3. *Arrows*, phosphorylated bcl-2 protein. PC3 cells were treated with DMSO or 1–10 μM taxol for 24 h in a humidified 5%-CO_2 incubator at 37°C. The immunoblot of the total protein extract was done as described previously (15). *B*, time course studies with 10 μM taxol for 1–24 h. *C*, another prostate cancer cell line, LNCaP, undergoes phosphorylation of *bcl-2* following taxol exposure. *D*, both okadaic acid and taxol can induce the phosphorylation of bcl-2 in PC3 cells.

It has been proposed that *bcl-2* protects cells from programmed cell death by dimerizing with *bax* and by protecting the cells from the apoptotic effect of *bax* homodimers (16, 17). It is possible that phosphorylation of bcl-2 (15) may interfere with its dimerization to *bax*, leading to more *bax* homodimers and death. To address this question, we performed co-immunoprecipitation experiments using antibodies against bcl-2 and bax proteins, as shown in Fig. 4. PC3 and LNCaP cells express *bax*, but DU145 did not (Fig. 4*A*). Cell extracts prepared from taxol-treated PC3 cells were immunoprecipitated with antibody against bcl-2. The immunocomplex was subsequently transferred on nitrocellulose and subjected to Western blot using anti-bax antibody. Fig. 4*B* clearly indicates more than 50% reduction of *bax* expression in the immunocomplexes immunoprecipitated by bcl-2 antibody. The experiment indicates that phosphorylated *bcl-2* is incapable of forming heterodimers with *bax*. Our results are consistent with previous reports (18) that describe that 50% reduction in the formation of *bcl-2/bax* heterodimer can drive cells toward apoptosis. We did not observe any quantitative and qualitative changes in *bax* in taxol-treated prostate cancer cells (data not shown). No modifications of bax protein were evident in prostate cancer cells by immunoblotting following taxol exposure (data not shown).

The results presented in this study indicate that the treatment of prostate cancer cells expressing *bcl-2* results in the phosphorylation of bcl-2 and in programmed cell death of the cancer cells concomitantly with a reduction of heterodimer complexes with *bax*. It has been speculated that the action of taxol on cancer cells results in the stabilization of microtubules (19). Our results indicate that taxol has other effects and may exercise its anticancer action through phosphorylation of bcl-2. The stabilization of microtubules and bcl-2 phos-

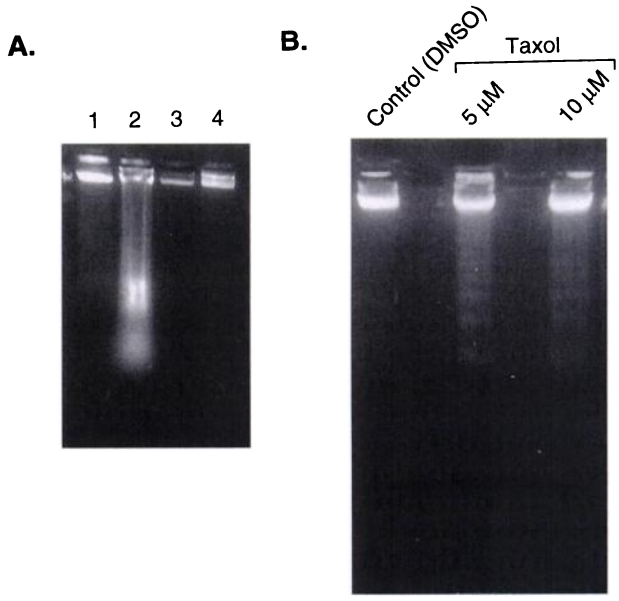

Fig. 2. *bcl-2*-expressing prostate tumor cells undergo apoptosis following taxol exposure. *A* and *B*, agarose gel of total DNA isolated from prostate cancer cells following DMSO or taxol treatment. *Lanes 1* and 2 of *A* contain DNA isolated from *bcl-2*-expressing LNCaP cells, whereas *Lanes 3* and *4* contain DNA isolated from *bcl-2*-negative prostate tumor cells DU145. *Lanes 1* and *3*, DMSO; *Lanes 2* and *4*, cells treated with 10 μM taxol for 48 h. *B*, total DNA isolated from *bcl-2*-positive prostate cancer cells PC3. The DNA was isolated from PC3 cells following 48 h DMSO or 10 μM taxol exposure. DNA fragmentation typical of apoptosis was clearly evident in *bcl-2*-expressing prostate cancer cells following taxol treatment (*Lane 2, A; Lanes 2* and *3, B*).

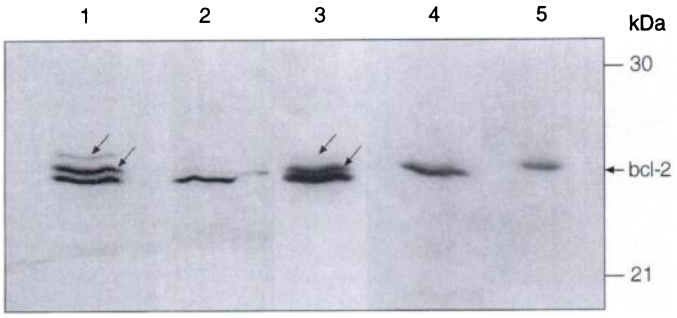

Fig. 3. Phosphorylated bcl-2 is enriched in microsomal fractions. PC3 prostate cancer cells were treated with 10 μM taxol for 24 h. Subsequently, subcellular fractionation was carried out by the method described earlier (5). The purity of the organelle was assessed by determining the activities of organelle marker enzymes, as described previously (5). *Lane 1,* total cellular extract; *lane 2,* nuclear fraction; *lane 3,* microsomal fraction; *lane 4,* mitochondria; *lane 5,* plasma membrane.

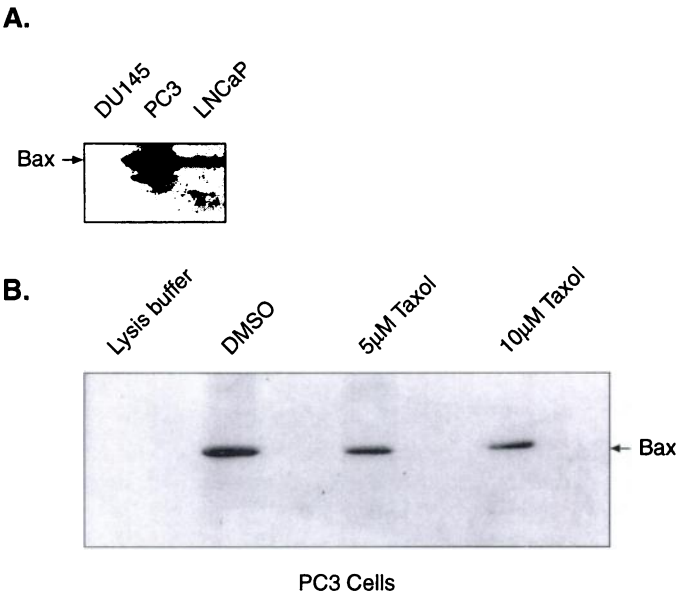

Fig. 4. Phosphorylated bcl-2 cannot form a heterodimer with *bax*. *A,* prostate cancer cells PC3 and LNCaP express bax protein. Immunoblot analysis of total cellular extract isolated from prostate cancer cells DU145, PC3, and LNCaP using a peptide antibody against *bax*. Peptide antibody was developed by immunizing the rabbit with synthetic peptide from the 44–66 amino acid region of the human bax protein (20). *B,* lesser extent of bax protein in the immunocomplex isolated from taxol-treated PC3 cells by bcl-2 antibody. Equal numbers of cells were treated with DMSO, 5 μM, and 10 μM taxol for 24 h, respectively. Lysates with equivalent amounts of protein were immunoprecipitated with bcl-2 monoclonal antibody by the method described earlier (20). Immunocomplexes were transferred on nitrocellulose, and Western blot was performed with peptide antibody against *bax*. Mock immunoprecipitation was carried out using lysis buffer.

phorylation may be, however, the consequences of the interaction of taxol with the same target, perhaps through the stimulation of a serine protein kinase. The fact that we observed the phosphorylated form of bcl-2 predominantly in the microsomal fraction of the prostate cancer cells suggests that the apoptotic effect of the phosphorylated form of bcl-2 (triggering of apoptosis) occurs at this subcellular location.

Our findings that prostate cancer cells that express *bcl-2* are sensitive to the apoptotic action of taxol suggest that the response of prostate cancers to taxol may depend on their *bcl-2* expression. Thus, these findings may lead to a more effective and rationale approach to the treatment of prostate cancer. Similarly, it will be important to determine whether other human neoplasms with *bcl-2* overexpression, such as follicular lymphoma, are sensitive to the apoptosis-inducing action of taxol.

Acknowledgments

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